

- (SEQ ID NO: 1) Gln<sup>59</sup>-Met-Glu-Ala-Glu-Ser(P)-Ile-Ser(P)-Ser(P)-Ser(P)-Glu-Ile-Val-Pro-Asn-Ser(P)-Val-Glu-Gln-Lys<sup>79</sup>.  $\alpha_{s1}(59-79)$
- (SEQ ID NO: 2) Arg<sup>1</sup>-Glu-Leu<sup>8</sup>-Glu-Ile-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Ser(P)-Leu-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-Ser-Ile-Thr-Arg<sup>25</sup>.  $\beta(1-25)$
- (SEQ ID NO: 3) Asn<sup>46</sup>-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-Ser(P)-Ala-Glu-Val-Ala-Thr-Glu-Glu-Val-Lys<sup>70</sup>.  $\alpha_{s2}(46-70)$
- (SEQ ID NO: 4) Lys<sup>1</sup>-Asn-Thr-Met-Glu-His-Val-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-Ser-Ile-Ile-Ser(P)-Gln-Glu-Thr-Tyr-Lys<sup>21</sup>.  $\alpha_{s2}(1-21)$

On page 4, delete the 1st paragraph, and replace this paragraph with the following in accordance with 37 CFR §1.121. A marked up version showing changes is attached:

In one aspect, the invention provides a stable calcium phosphate complex, comprising amorphous calcium phosphate or a derivative thereof stabilized by a phosphopeptide, wherein said phosphopeptide comprises the sequence Ser(P)-Ser(P)-Ser(P)-Glu-Glu-(SEQ ID NO: 5).

On page 4, delete the 3<sup>rd</sup> paragraph, and replace this paragraph with the following in accordance with 37 CFR §1.121. A marked up version showing changes is attached:

The phosphopeptide (PP) may be from any source; it may be obtained by tryptic digestion of casein or other phospho-acid rich proteins such as phosphitin, or by chemical or recombinant synthesis, provided that it comprises the core sequence - Ser(P)-Ser(P)-Ser(P)-Glu-Glu-(SEQ ID NO: 5). The sequence flanking this core sequence may be any sequence. However, those flanking sequences in  $\alpha_{s1}(59-79)$  (SEQ ID NO: 1),  $\beta(1-25)$  (SEQ ID NO: 2),  $\alpha_{s2}(46-70)$  (SEQ ID NO: 3) and  $\alpha_{s2}(1-21)$  (SEQ ID NO: 4) are preferred. The flanking sequences may optionally be modified by deletion, addition or conservative substitution of one or more residues. The amino acid composition and sequence of the flanking region are not critical as long as the conformation of the peptide is maintained and that all phosphoryl and carboxyl groups interacting with

C3 calcium ions are maintained as the preferred flanking regions appear to contribute to the structural action of the motif.

On page 12, delete the 2<sup>nd</sup> paragraph, and replace this paragraph with the following in accordance with 37 CFR §1.121. A marked up version showing changes is attached:

C4 A 10% w/v casein (Murray Goulburn, Victoria, Australia) or caseinate solution was prepared at pH 8.0 and then digested with trypsin at 0.2% w/w of the casein for 2h at 50°C with the pH controlled to  $8.0 \pm 0.1$  by NaOH addition. After digestion the solution was adjusted to pH 4.6 by the addition of HCl and the precipitate removed by centrifugation or microfiltration. However, the solution can also be clarified by microfiltration at pH 8.0 without acidification. The supernatant or microfiltrate was then adjusted to pH 9.0 with NaOH, then  $\text{CaCl}_2$  (1.6 M) and  $\text{Na}_2\text{HPO}_4$  (1 M) at pH 9.0 were added slowly ( $\leq 1\%$  vol per min) with constant agitation with the pH held constant at  $9.0 \pm 0.1$  by NaOH addition.  $\text{CaCl}_2$  and sodium phosphate were added to the final concentrations of 100 mM and 60 mM respectively. Following the addition of the calcium and phosphate solutions, the solution was microfiltered through a 0.1 or 0.2  $\mu\text{m}$  microfilter (ceramic or organic) to concentrate the solution five fold. The retentate was then diafiltered with one to five volumes of distilled water. The retentate after diafiltration was spray-dried to produce a white powder that was 50% CPP and 40% ACP and residue water. Analysis of the CPP of the CPP-ACP complex by reversed-phase HPLC, sequence analysis and mass spectrometry revealed that the only peptides that are capable of stabilizing the amorphous calcium phosphate and retained during the microfiltration and diafiltration are Bos  $\alpha_{s1}$ -casein X-5P (f59-79) (SEQ ID NO: 1), Bos  $\beta$ -casein X-4P (f1-25) (SEQ ID NO: 2), Bos  $\alpha_{s2}$ -casein X-4P (f46-70) (SEQ ID NO: 3) and Bos  $\alpha_{s2}$ -casein X-4P (f1-21) (SEQ ID NO: 4) and truncated and heat modified forms of these peptides.

On page 13-14, delete the 4<sup>th</sup> paragraph, and replace this paragraph with the following, in accordance with 37 CFR §1.121 (a marked up version is attached which shows the changes):

Casein phosphopeptides containing the Ser(P) cluster, i.e. the core sequence motif Ser(P)-Ser(P)-Ser(P)-Glu-Glu-(SEQ ID NO: 5), have a marked ability to stabilize calcium phosphate in solution. Solutions containing 0.1% w/v  $\alpha_{s1}$ (59-79) (SEQ ID NO: 1) at various pH, calcium and phosphate concentrations, but constant ionic strengths were used to characterize the peptide's interaction with calcium phosphate. The peptide was found to maximally bind 24 Ca and 16 Pi per molecule as shown in Table 1.

On page 14, delete the 1<sup>st</sup> paragraph, and replace this paragraph with the following in accordance with 37 CFR §1.121. A marked up version showing changes is attached:

The ion activity products for the various calcium phosphate phases [hydroxyapatite (HA); octacalcium phosphate (OCP); tricalcium phosphate (TCP); amorphous calcium phosphate (ACP); and dicalcium phosphate dihydrate (DCPD) were determined from the free calcium and phosphate concentrations at each pH using a computer program that calculates the ion activity coefficients through the use of the expanded Debye-Hückel equation and takes into account the ion pairs  $\text{CaHPO}_4^0$ ,  $\text{CaH}_2\text{PO}_4^+$ ,  $\text{CaPO}_4^-$  and  $\text{CaOH}^+$  the dissociation of  $\text{H}_3\text{PO}_4$  and  $\text{H}_2\text{O}$  and the ionic strength. The only ion activity product that significantly correlated with calcium phosphate bound to the peptide independently of pH was that corresponding to ACP  $[\text{Ca}_3(\text{PO}_4)_{1.87}(\text{HPO}_4)_{0.02}\text{H}_2\text{O}]$  indicating that this is the phase stabilized by  $\alpha_{s1}$ (59-79) (SEQ ID NO: 1). The peptide  $\alpha_{s1}$ (59-79) (SEQ ID NO: 1) binds to forming ACP clusters producing a metastable solution preventing ACP growth to the critical size required for nucleation and precipitation. The binding of  $\alpha_{s1}$ (59-79) (SEQ ID NO: 1) to ACP results in the formation of colloidal complexes with the unit formula  $[\alpha_{s1}(59-79)(\text{SEQ ID NO: 1})(\text{ACP})_n]_n$  where n is equal to or greater than one. It is likely that the predominant form is  $n = 6$  as  $\alpha_{s1}$ (59-79) (SEQ ID NO: 1) cross-linked with glutaraldehyde in the presence of ACP runs as a hexamer on polyacrylamide gel electrophoresis. Interestingly, the synthetic octapeptide  $\alpha_{s1}$ (63-70) AcGlu-Ser(P)-Ile-Ser(P)-Ser(P)-Ser(P)-Glu-GluNHMe (SEQ ID NO: 6) only binds 12 Ca and 8 Pi per molecule i.e.  $(\text{ACP})_4$  and the synthetic peptides corresponding to the N-terminus  $\alpha_{s1}$ (59-63), Gln-Met-Glu-Ala-Glu (SEQ ID NO: 7) and the C-terminus  $\alpha_{s1}$ (71-78), Ile-Val-Pro-Asn-Ser(P)-Val-Glu-Gln (SEQ ID NO: 8) of

CF  $\alpha_{s1}$ (59-79) did not bind calcium phosphate as shown in Table 1. These results indicate that conformational specificity is essential for full ACP binding.

On page 15, delete the 2<sup>nd</sup> paragraph, and replace this paragraph with the following in accordance with 37 CFR §1.121. A marked up version showing changes is attached:

CF We have demonstrated medium- and long-range nuclear Overhauser enhancements (nOes) in 2D <sup>1</sup>H NMR spectra of  $\alpha_{s1}$ (59-79) (SEQ ID NO: 1) in the presence of Ca<sup>2+</sup> indicating a conformational preference. Two structured regions were identified. Residues Val72 to Val76 are implicated in a  $\beta$ -turn conformation. Residues Glu61 to Ser(P)67, which extend over part of the Ser(P) cluster motif -Ser(P)-Ser(P)-Ser(P)-Glu-Glu- (SEQ ID NO: 5) are involved in a loop-type structure. 2D NMR studies on  $\beta$ -casein(1-25) (SEQ ID NO: 2) in the presence of calcium have shown a medium range nOe in the -Ser(P)<sup>17</sup>-Ser(P)-Ser(P)-Glu-Glu<sup>21</sup>- (SEQ ID NO: 5) motif region between the C $\alpha$ H of Ser(P)<sup>18</sup> and NH of Blu<sup>20</sup>. Further medium range nOes include one between the C $\alpha$ H of Ser<sup>22</sup> and NH of Thr<sup>24</sup>. Evidence from the <sup>1</sup>H NMR spectra of  $\alpha_{s2}$ -casein(1-21) [4] have shown that several residues including those around the -Ser(P)-Ser(P)-Ser(P)-Glu-Glu- (SEQ ID NO: 5) are perturbed. Furthermore, there are medium range nOes between NH of Ser(P)<sup>8</sup> and NH of GLU<sup>10</sup>. This is yet another example of a medium range nOe in the -Ser(P)-Ser(P)-Ser(P)-Glu-Glu- (SEQ ID NO: 5) motif. Other examples of medium range nOes include that between the NH of Ile<sup>14</sup> and NH of Ser(P)<sup>16</sup>.

On page 16, delete the 1<sup>st</sup> paragraph, and replace this paragraph with the following in accordance with 37 CFR §1.121. A marked up version showing changes is attached:

CS In summary the NMR data indicates that preferred conformations exist for these peptides in the presence of calcium ions. Molecular modeling of both  $\alpha_{s1}$ (59-79) (SEQ ID NO: 1) and  $\beta$ (1-25) (SEQ ID NO: 2) using the constraints derived from the NMR spectroscopy have indicated that the peptides adopt conformations that allow both

*C8 ant*  
 glutarnyl and phosphoseryl side chains of the cluster motif –Ser(P)-Ser(P)-Ser(P)-Glu-Glu (SEQ ID NO: 5) to interact collectively with calcium ions of the ACP.

On page 16, delete the 2<sup>nd</sup> paragraph, and replace this paragraph with the following in accordance with 37 CFR §1.121 (see attached mark-up version):

*C9*  
 The relationship between CPP structure and interaction with amorphous calcium phosphate was investigated using a series of synthetic peptide homologues and analogues indicated in Table 1. These studies showed that the cluster sequence-Ser(P)-Ser(P)-Ser(P)-Glu-Glu- (SEQ ID NO: 5) was mainly responsible for the interaction with ACP and that all three contiguous Ser(P) residues are required for maximal interaction with ACP.

On page 17, replace Table 1 with the following table:

TABLE 1

### Calcium Phosphate Binding by CPP and Synthetic Homologues and Analogues

	V <sub>ca</sub> mol/mol	V <sub>pi</sub> mol/mol	Ca/P
(SEQ ID NO: 5)ΣΣΣEE	9	6	1.5
(SEQ ID NO: 9)SΣΣEE	2	1	2.0
(SEQ ID NO: 10)EΣΣEE	2	1	2.0
(SEQ ID NO: 11)DΣΣEE	2	1	2.0
(SEQ ID NO: 12)000EE	9	6	1.5
(SEQ ID NO: 13)S00EE	2	1	2.0
(SEQ ID NO: 14)AΣAE	0	0	
(SEQ ID NO: 15)IAΣAEA	0	0	
(SEQ ID NO: 16)EAIAΣAEA	0	0	
(SEQ ID NO: 17)AΣAΣAE	0	0	
(SEQ ID NO: 18)AΣAΣAΣAE	2	1	1.5
(SEQ ID NO: 19)AΣAΣAΣAΣAE	6	4	1.5
(SEQ ID NO: 1)α <sub>s1</sub> (59-79) QMEAEΣIΣΣΣEEIVPNΣVEQK	24	16	1.5
(SEQ ID NO: 6)α <sub>s1</sub> (63-70) EΣIΣΣΣEE	12	8	1.5
(SEQ ID NO: 5)α <sub>s1</sub> (66-70) ΣΣΣEE	9	6	1.5
(SEQ ID NO: 8)α <sub>s1</sub> (71-78) IVPNΣVEQ	0	0	

CP  
(SEQ ID NO: 7) $\alpha_{s1}$ (59-63) QMEAE  
(SEQ ID NO: 2) $\beta$ (1-25)  
RELEELNVPGEIVE $\Sigma$ L $\Sigma$  $\Sigma$ EEESITR  
(SEQ ID NO: 20) $\beta$ (14-21) E $\Sigma$ L $\Sigma$  $\Sigma$ EE

0	0	
24	16	1.5
12	8	1.5

$\Sigma$  = Ser(P),  $\theta$  = Thr(P), E = Glu, D = Asp, S = Ser, A = Ala, I = Ile, Q = Gln, M = Met, V = Val, P = Pro, K = Lys, L = Leu, T = Thr, G = Gly and R = Arg.

On page 19, replace Table 2 with the following:

TABLE 2.

CPP and Synthetic Peptide binding to HA at 37°C

CP  
MMA

	K ml/ $\mu$ mol	N $\mu$ mol/ m <sup>2</sup>	Molecular Area nm <sup>2</sup>
(SEQ ID NO: 1) $\alpha_{s1}$ (59-79) QMEAE $\Sigma$ I $\Sigma$ $\Sigma$ $\Sigma$ EEIVPN $\Sigma$ VEQK	415	0.35	4.75
(SEQ ID NO: 6) $\alpha_{s1}$ (63-70) E $\Sigma$ I $\Sigma$ $\Sigma$ EE	10,370	0.47	3.56
(SEQ ID NO: 5) $\alpha_{s1}$ (66-70) $\Sigma$ $\Sigma$ $\Sigma$ EE	12,845	0.52	3.27
(SEQ ID NO: 8) $\alpha_{s1}$ (71-78) IVPN $\Sigma$ VEQ	-	-	-
(SEQ ID NO: 7) $\alpha_{s1}$ (59-63) QMEAE	-	-	-
(SEQ ID NO: 5) $\Sigma$ $\Sigma$ $\Sigma$ EE	12,845	0.52	3.27
(SEQ ID NO: 10)E $\Sigma$ $\Sigma$ EE	1,513	0.96	1.74
(SEQ ID NO: 11)D $\Sigma$ $\Sigma$ EE	6,579	0.81	2.04
(SEQ ID NO: 12) $\theta$ $\theta$ $\theta$ EE	12,234	0.51	3.27
(SEQ ID NO: 21)T $\theta$ $\theta$ EE	1,013	0.55	3.03
(SEQ ID NO: 22) $\theta$ T $\theta$ EE	837	0.44	3.77
(SEQ ID NO: 23) $\theta$ $\theta$ TEE	1,799	0.46	3.61

$\Sigma$  = Ser(P),  $\theta$  = Thr(P)

On page 20, delete the second paragraph with the following, in accordance with 37 CFR §1.121. A marked up version showing changes is attached:

C12  
We have also studied the docking of the peptide Ser(P)- Ser(P)- Ser(P)-Glu-Glu- onto three crystallographic planes of HA, {100}, {010} and {001} using computer simulation techniques and the unit cell coordinates of synthetic HA. These simulation studies revealed that the peptide - Ser(P)- Ser(P)- Ser(P)-Glu-Glu- is more likely to the {100} surface, followed by the {010} surface. The Ser(P)- cluster motif can therefore bind to both {100} and {010} surfaces thus allowing deposition of calcium, phosphate and hydroxyl ions on the {100} surface enabling growth of the HA crystal along the c-axis only. These results therefore can now explain the c-axis growth of HA crystals in enamel and dentine. Detailed examination of the computer simulation data shows that the - Ser(P)- Ser(P)- Ser(P)-Glu-Glu- conformer with the greatest relative binding energy is positioned on the HA surface such that the carboxyl groups of the glutamyl residues and the phosphoryl groups of the phosphoserine residues are in proximity to the HA surface with maximal contact between these groups and surface calcium atoms.

On page 21, replace the first paragraph with the following, in accordance with 37 CFR §1.121. A marked up version showing changes is attached:

C13  
Two exposures of the CPP-ACP solution per day to the right pair of enamel slabs for 12 subjects produced a  $51\% \pm 19\%$  reduction in enamel mineral loss relative to the left-side, control enamel. The plaque exposed to the CPP-ACP solution contained  $78 \pm 22$   $\mu\text{mol/g}$  calcium,  $52 \pm 25$   $\mu\text{mol/g}$   $P_i$  and  $2.4 \pm 0.7$  mg/g CPP compared with  $32 \pm 12$   $\mu\text{mol/g}$  calcium and  $20 \pm 11$   $\mu\text{mol/g}$   $P_i$  in the control plaque. The level of the CPP was determined by competitive ELISA using an antibody that recognizes both  $\alpha_{s1}(59-79)$  and  $\beta(1-25)$ . Electron micrographs of immunocytochemically stained sections of the plaque revealed localization of the peptide predominantly on the surface of microorganisms but also in the extracellular matrix.

#### IN THE CLAIMS

Please cancel claim 29, without prejudice or disclaimer.

In accordance with 37 CFR §1.121, please substitute, for claims 1, 4, 5, 7, 9, 11, 12 and 13, the following rewritten versions of the same claims. The changes proposed are detailed in the attached "Version with Markings to Show Changes Made."